EFFECTS OF MODIFICATION OF ε-AMINO GROUPS ON THE INTERACTION OF κ- AND α_{s1}-CASEINS

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SUMMARY

1. The reaction of κ-casein B and α_{s1}-casein C with formaldehyde suggested that their free amino groups might be essential for their native functions. Other amino group-modifying reagents were then employed since the specificity of formaldehyde is doubtful.

2. The capacity of κ-casein B for stabilizing α_{s1}-casein C in the presence of Ca^{2+} is abruptly abolished after five of its nine positively charged lysine residues per molecule (monomer mol. wt., 19 000) are converted to uncharged homocitrulline residues by carbamylation. A marked decrease in sedimentation coefficient at 1% protein concentration coincided with the loss of protective colloid function of carbamylated κ-casein B. These findings indicate that changes in conformation and/or aggregation of κ-casein B occur due to the increase in net negative charge that accompanied carbamylation of the fifth lysine residue per molecule.

3. In other experiments the lysine residues of κ- and α_s-caseins were converted to homoarginine, ε-*N*,*N*-dimethyllysine and ε-*N*-isopropyllysine residues. Such modification retains the charges of the lysine residues while sterically hindering their ε-amino groups. Since the native properties of the κ-casein B and α_{s1}-casein C were conserved, it seems doubtful that a specific ε-amino group is critically involved in the interaction of these proteins.

INTRODUCTION

WAUGH AND VON HIPPEL[1] first demonstrated that a component of the casein micelles of cow's milk, named κ-casein, possesses the protective colloid function which maintains micellar stability. They also showed that κ-casein, a glycoprotein, is soluble in dilute Ca^{2+} solution and is the substrate for rennin, a milk-clotting enzyme. Subsequently, WAKE[2] reported the isolation of κ-casein in relatively pure form. Other investigators elucidated the genetic variability, composition, structure and interactions of this protein. These investigations concerning κ-casein have been summarized in the review article by MCKENZIE[3]. Also discussed in this review is the isolation, purification, composition and genetic variation of the Ca^{2+}-sensitive α_{s1}- and β-caseins

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which are stabilized by κ-casein in the presence of Ca^{2+} and are constituents of the micelles which exist in milk. The prevailing view emerging from the accumulated information is that the stable casein micelle structure is maintained by hydrophobic interactions, non-specific electrostatic forces, and calcium phosphate salt bridges.

However, experience accumulated in this laboratory from the formaldehyde hardening of casein plastics, and evidence that free amino groups are involved in the reaction[4,5] suggested that study of the interaction of κ- and α_{s1}-caseins by chemical modification of these groups might be fruitful.

Preliminary experiments were performed at low protein concentration, where the major reaction with formaldehyde has been reported to result in *N*-methylol derivatives of the free amino groups without crosslinking of other protein groups through methylene bridges[6]. The changes in properties that occurred indicated that it would be worthwhile to pursue the study further with reagents that react irreversibly and more specifically with the ε-amino groups of proteins than formaldehyde does. To this end, κ- and α_{s1}-caseins were carbamylated, guanidinated, and reductively alkylated, and the interaction of the modified proteins was investigated.

MATERIALS AND METHODS

α_{s1}-Casein C was prepared by the method of THOMPSON AND KIDDY[7] from the milk of a cow homozygous for this genetic variant. The κ-casein was prepared from the milk of a cow typed κ-B by the method of MCKENZIE AND WAKE[8]. Experiments were also performed with κ-casein of the B type furnished by Dr. J. H. Woychik and by Dr. C. A. Zittle and J. H. Custer* of this laboratory, which were prepared by the latter's method[9]. Homogeneity of the proteins was established by polyacrylamide gel electrophoresis. The amino acid compositions of the κ-casein B and α_{s1}-casein C were identical to those reported by KALAN AND WOYCHIK[11], and by GORDON *et al.*[12], respectively.

The reaction of formaldehyde with the caseins was performed in 0.01 M imidazole buffer (pH 7.0) at concentrations of 0.25% α_{s1}-C and 0.125% κ-B. Each protein was reacted at 27 and at 60° and at formaldehyde concentrations of 0.25 and 0.125%. Aliquots of the reaction mixtures were taken at various times and the stability of the α_{s1}-casein and the stabilizing capacity of κ-casein in dilute $CaCl_2$ at neutral pH were determined. The presence of formaldehyde did not interfere with the determination of the Ca^{2+} stability of α_{s1}-casein or the stabilizing capacity of κ-casein. Details of the test conditions are presented in a subsequent paragraph.

Carbamylation of the κ-casein amino groups was carried out in solution at 50°, 0.1 M KNCO (pH 7.0), and 1.0% protein concentration. Aliquots were removed at 0, 15, 30, 45, 60, 90, 120, 210 min and 16 h, dialyzed against distilled water for 48 h at 4° and then lyophilized after adjusting the pH to 7.5. The stabilizing capacity of the lyophilized κ-casein derivatives was measured and correlated with the number of lysine residues per molecule that were converted by carbamylation to homocitrulline. The homocitrulline was determined according to STARK *et al.*[13]. The carbamylated derivatives of κ-casein were digested with rennin and the split products,

* In preliminary reports[10] evidence was presented for the specific involvement of one or more lysine residues of κ-casein B in its interaction with α_{s1}-casein C. The behavior of that κ-casein preparation might have been abnormal as sometimes occurs with this protein.

a glycopeptide and a much larger peptide named para-κ-casein, were separated in 12% trichloroacetic acid, dialyzed, lyophilized and analyzed in order to determine the distribution of homocitrulline residues between the fragments.

α_{s1}-Casein was also carbamylated under the above conditions for 16 h, dialyzed free of reagents, lyophilized after adjusting the pH to 7.5, and its stability in 0.04 M $CaCl_2$ was measured.

Guanidination of the proteins was performed with *O*-methylisourea according to the method of ROCHE *et al.*[14], but in the presence of 8 M urea and 0.01 M β-mercaptoethanol. After dialysis for 48 h at 4° against distilled water and lyophilization from solutions adjusted to pH 7.5 the guanidinated derivatives of κ-casein and α_{s1}-casein were tested for protective colloid capacity and Ca^{2+} stability, respectively. The extent of the conversion of the lysyl residues to homoarginyl residues was measured by amino acid analysis. Homoarginine is eluted about 90 min after the arginine peak on the 15-cm column of the amino acid analyzer using a flow rate of 40 ml/h, increased from the original 30 ml/h.

Reductive alkylation of the κ- and α_{s1}-casein amino groups was carried out according to the method of MEANS AND FEENEY[15], also in the presence of 8 M urea and 0.01 M β-mercaptoethanol. With formaldehyde and sodium borohydride the ε-*N*,*N*-dimethyllysine derivatives of the caseins were obtained, whereas reaction with acetone and sodium borohydride under similar conditions yielded the ε-*N*-isopropyllysine derivatives. The modified proteins were dialyzed at 4° for 48 h and lyophilized from solutions adjusted to pH 7.5 with dilute NaOH. The protective colloid function of the alkylated κ-casein and the stability of the alkylated α_{s1}-casein in 0.04 M $CaCl_2$ (pH 6.7–7.0) were determined. The extent of amino group modification was estimated by the decrease in lysine found upon amino acid analysis.

The stabilization test was performed by adding 1.0 ml of a 0.125% solution of the various lyophilized κ-casein derivatives to 2.0 ml of a 0.25% α_{s1}-casein solution (pH 7.8) and diluting to 4.0 ml with water. 4 ml of 0.08 M $CaCl_2$ is blown in rapidly with vigorous magnetic stirring and the solutions, final pH 6.7–7.0, are left at room temperature for 0.5 h and then centrifuged under a force of $3000 \times g$ for 10 min. After clarification of the supernatant with a drop of 1 M NaOH, its absorbance is measured at 280 mμ in a Beckman DU spectrophotometer*. The concentration of α_{s1}-casein, stabilized by the modified κ-caseins, is obtained using $E^{1\%}_{1cm} = 10.0$ (see ref. 9) for α_{s1}-casein C and 12.2 (see ref. 11) for κ-casein B. The stability of the α_{s1}-casein derivatives was determined under the same conditions as above but in the absence of κ-casein. The derivatives of α_{s1}-casein which retained their sensitivity to Ca^{2+} were also tested for their capacity to be stabilized by native κ-casein.

κ-Casein and its derivatives were digested with rennin as described by KALAN AND WOYCHIK[11].

Polyacrylamide gel electrophoresis was performed on the κ- and α_{s1}-casein derivatives according to PETERSON[16].

The degree of modification of the protein was determined by amino acid analysis on 24-h hydrolysates according to MOORE AND STEIN[17]. The reductively alkylated derivatives of lysine were resolved on the 15-cm column by the MOORE AND STEIN[17] method without any changes in the eluting buffer system[15].

* Mention of companies or products is for the convenience of the reader and does not constitute an endorsement by the U.S. Department of Agriculture.

Sedimentation coefficients were obtained in a Spinco Model E ultracentrifuge by Dr. R. M. Parry, Jr. and R. O. Deakyne. The conditions were 1.0% protein, 0.05 M cacodylate buffer (pH 7.0), 0.15 M NaCl, 25°, 59 780 rev./min.

RESULTS AND DISCUSSION

Fig. 1 shows the increasing stability of α_{s1}-casein in 0.04 M $CaCl_2$ (pH 7.0) as reaction with formaldehyde proceeds, the rate of reaction being an increasing function of the formaldehyde concentration and the temperature. In fact, no visible opalescence characteristic of micelle formation was observed in a Ca^{2+} solution of modified α_{s1}-casein samples taken at 24 h at 60°.

In Fig. 2 one observes the progressive decrease in the ability of formaldehyde reacted κ-casein to stabilize native α_{s1}-casein against precipitation in 0.04 M $CaCl_2$.

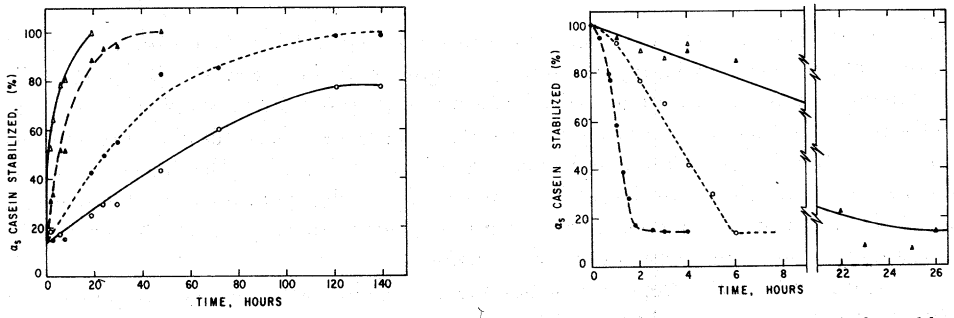

Fig. 1. Per cent α_{s1}-C casein stable to 0.04 M $CaCl_2$ (pH 7.0) *versus* reaction time with formaldehyde. △—△, 60°, 0.25% formaldehyde; ▲— — —▲, 60°, 0.125% formaldehyde; ●— — —●, 27°, 0.25% formaldehyde; ○—○, 27°, 0.125% formaldehyde.

Fig. 2. Stabilizing capacity of κ-B casein, expressed as per cent α_{s1}-C casein stabilized in 0.04 M $CaCl_2$ (pH 7.0), *versus* reaction time of κ-casein B with formaldehyde. ●— —●, 60°, 0.25% formaldehyde; ○— — —○, 60°, 0.125% formaldehyde; △—△, 27°, 0.25% formaldehyde.

It appeared, from the experiments at low protein concentrations at 27°, where amino groups have been reported[6] to be modified without crosslinking to other protein groups, that these amino groups are specifically involved in the self-association and interaction of the two caseins. This conclusion seemed hasty upon closer inspection because the immediate reaction of protein amino groups with formaldehyde forms *N*-methylol derivatives at room temperature, but changes in the properties of the caseins were not immediately observed. The reaction times at which changes in the properties of the caseins appeared, even at 27°, suggested that more complex, non-specific reactions with formaldehyde were occurring. This situation led us to try carbamylation of the casein amino groups, since this reaction was shown to be specific for protein amino groups under controlled experimental conditions[13].

As a result of reaction with KNCO the sixteen lysine residues of α_{s1}-casein C were converted to homocitrulline and the protein became stable to Ca^{2+}. The N-terminal α-amino group probably was carbamylated also, but the product is unstable during hydrolysis preceding amino acid analysis. As with the *N*-methylol derivative of α_{s1}-casein, the carbamylated derivative of α_{s1}-casein developed very little opale-

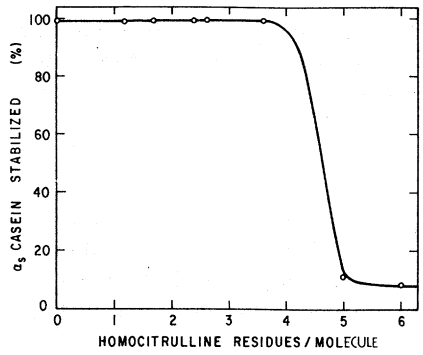

Fig. 3. Stabilizing capacity of carbamylated κ-B casein, expressed as per cent α_{s1}-C stabilized in 0.04 M $CaCl_2$ (pH 7.0), *versus* extent of carbamylation, expressed as homocitrulline residues per molecule of κ-B.

scence in the presence of Ca^{2+}. The failure of the *N*-methylol and carbamylated derivatives of α_{s1}-casein to aggregate and to precipitate in the presence of 0.04 M $CaCl_2$ is explained by the increased non-specific electrostatic repulsion between the molecules of the derivatives as a result of their increased net negative charge. Similar observations were reported for acetylated and succinylated β-casein A by HOAGLAND[18]. These chemical modifications also increase the net negative charge of β-casein A molecules by abolishing positive charges of protein amino groups, thus reducing Ca^{2+} sensitivity and aggregation through electrostatic repulsion.

The results of the step-wise carbamylation studies with κ-casein are plotted in Fig. 3. It is clear that no change in the stabilizing capacity of κ-casein is found until five of the nine lysine residues per molecule of κ-casein B are converted to homocitrulline*. At this point κ-casein abruptly loses its ability to stabilize α_{s1}-casein against precipitation by Ca^{2+}. Amino acid analysis of the glycopeptide and para-κ-casein fragments resulting from digestion of the carbamylated derivatives with rennin, located the fifth lysine residue in the glycopeptide portion of the κ-casein molecule.

Polyacrylamide gel electrophoresis at pH 8.7 of the derivatives in the presence of β-mercaptoethanol gave patterns similar to that for reduced native κ-casein[3] but with increased mobilities of the bands. This is consistent with the increase in net charge resulting from removal of positive charges at the free amino groups of κ-casein. That no proliferation of the original number of bands results upon gel electrophoresis of the successively carbamylated derivatives suggests that the molecules were rather uniformly modified.

The sedimentation coefficients ($s_{25,w}$) for the series of carbamylated derivatives of κ-casein remained constant at 14.2, the value for native κ-casein, until a sharp decrease to 11.3 was observed for the κ-casein derivative containing five homocitrulline residues per molecule. This coincides with the point at which κ-casein loses its protective colloid function.

The abrupt loss of the protective colloid function of κ-casein after a lysine

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* Reaction of the N-terminal α-amino group with amino group-specific reagents has not been demonstrated[19].

group in its glycopeptide region was converted to an uncharged homocitrulline residue, implies that the disruption of specific ionic linkages between κ-casein and α_{s1}-casein aggregates impairs their ability to interact. However, the change in the sedimentation coefficient at the point where the native properties of κ-casein are abolished by carbamylation indicates that significant alterations in aggregation and/or conformation of κ-casein had occurred. WOYCHIK[20] has blocked the ε-amino groups of κ-casein B with trifluoroacetyl groups and has obtained results similar to those presented above. This is not surprising since trifluoroacetylation of protein amino groups drastically alters the charge patterns of the proteins. The non-specific changes due to the increased net negative charge of the κ-casein molecules would have to be avoided before a claim could be made for the critical involvement of a specific ε-amino group of κ-casein B in its interaction with α_{s1}-casein C.

Evidence has been presented[21,22] that guanidination of proteins retains the charge pattern of the native protein and produces no significant conformational change. Similar evidence was presented in the case of reductive alkylation of proteins[15]. In our guanidination and reductive alkylation experiments the lysine residues of α_{s1}-casein were completely converted to homoarginine, ε-*N*,*N*-dimethyllysine and ε-*N*-isopropyllysine residues. The patterns obtained upon gel electrophoresis were the same as for native α_{s1}-casein. The three derivatives of α_{s1}-casein also retained the sensitivity to Ca^{2+} of the native protein: as with unmodified α_{s1}-casein, about 85% of each derivative was precipitated by Ca^{2+} under the conditions of the stabilization test described previously. Furthermore, these derivatives were stabilized by κ-casein in the presence of Ca^{2+}.

Completely guanidinated and reductively alkylated κ-casein derivatives were obtained by the methods employed for modification. Gel electrophoresis gave patterns identical to that obtained for native reduced κ-casein B. All three κ-casein derivatives were completely stable in 0.04 M $CaCl_2$ and also stabilized native α_{s1}-casein against precipitation by Ca^{2+}.

It must be noted that guanidination and reductive alkylation of the caseins were performed in 8 M urea and 0.01 M β-mercaptoethanol in order to achieve complete modification of their lysine residues, since several attempts failed in the absence of these denaturing agents.

It has been demonstrated[15] that the N-terminal α-amino group of some proteins can be reductively alkylated at the same rate as the ε-amino group. However, for the proteins employed in this study no alkylated amino acid other than lysine was found as judged by quantitative recovery of the other amino acids in amino acid analysis.

The conservation of the native properties of κ-casein B and α_{s1}-casein C after blocking their ε-amino groups, with retention of their positive charges, is evidence that specific ε-amino groups are probably not critical for the interactions and self-associations of the two proteins. It might still be doubted that blocking the casein ε-amino groups with guanidinium, ε-*N*,*N*-dimethyl, and ε-*N*-isopropyl groups would sterically hinder their interaction with negatively charged groups. Evidence cited previously[15,21,22] will largely dispel these doubts, since in these studies a number of proteins were modified by guanidination and reductive alkylation, and although no significant conformational changes could be detected, a loss of protein function occurred after a given number of amino groups were blocked. Furthermore, LEWIN[23] employing Courtaulds atomic models has attempted to define the rigorous steric

requirements that must be met in order for electrostatic bonding to be significant between positively charged nitrogenous groups and negatively charged carboxylate or phosphate groups. If his arguments are sound, blocking the ε-amino groups of the proteins employed here with groups of such bulk as guanidinium, dimethyl or isopropyl groups would significantly hinder the formation of ionic linkages in protein–protein interactions.

Thus, in sum, our evidence indicates that ε-amino groups of lysine are not specifically involved in the interaction and self-association of κ-casein B and α_{s1}-casein C, but that increased net negative charge and altered charge patterns in the carbamyl derivatives of the proteins are responsible for the drastic effects of carbamylation on the protective colloid function of κ-casein and on the sensitivity to Ca^{2+} of α_{s1}-casein.

REFERENCES

1 D. F. WAUGH AND P. H. VON HIPPEL, *J. Am. Chem. Soc.*, 78 (1956) 4576.
2 R. G. WAKE, *Australian J. Biol. Sci.*, 12 (1959) 538.
3 H. A. MCKENZIE, *Advan. Protein Chem.*, 22 (1967) 55.
4 H. FRAENKEL-CONRAT AND H. S. OLCOTT, *J. Am. Chem. Soc.*, 70 (1948) 2673.
5 H. FRAENKEL-CONRAT AND H. S. OLCOTT, *J. Biol. Chem.*, 174 (1948) 827.
6 H. FRAENKEL-CONRAT AND D. K. MECHAM. *J. Biol. Chem.*, 177 (1949) 477.
7 M. P. THOMPSON AND C. A. KIDDY, *J. Dairy Sci.*, 47 (1964) 626.
8 H. A. MCKENZIE AND R. G. WAKE, *Biochim. Biophys. Acta*, 47 (1961) 240.
9 C. A. ZITTLE AND J. H. CUSTER, *J. Dairy Sci.*, 46 (1963) 1183.
10 L. PEPPER, N. J. HIPP AND W. G. GORDON, *Am. Chem. Soc. Abstr., 156th Natl. Meeting, Atlantic City, N.J., 1968, Div. Biol. Chem.*, 15; *Am. Chem. Soc. Abstr., 3rd Middle Atlantic Regional Meeting, Philadelphia, Pa., 1968, Div. Med. and Biochem.*, G-34.
11 E. B. KALAN AND J. H. WOYCHIK, *J. Dairy Sci.*, 48 (1965) 423.
12 W. G. GORDON, J. J. BASCH AND M. P. THOMPSON, *J. Dairy Sci.*, 48 (1965) 1010.
13 G. R. STARK, W. H. STEIN AND S. MOORE, *J. Biol. Chem.*, 235 (1960) 3177.
14 J. ROCHE, M. MOURGUE AND R. BARET, *Bull. Soc. Chim. Biol.*, 36 (1954) 85.
15 G. E. MEANS AND R. E. FEENEY, *Biochemistry*, 7 (1968) 2192.
16 R. F. PETERSON, *J. Dairy Sci.*, 46 (1963) 1136.
17 S. MOORE AND W. H. STEIN, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. 6, Academic Press, New York, N.Y. 1963, p. 819.
18 P. D. HOAGLAND, *J. Dairy Sci.*, 49 (1966) 783.
19 R. G. WAKE, *Australian J. Biol. Sci.*, 12 (1959) 479.
20 J. H. WOYCHIK, *J. Dairy Sci.*, 52 (1969) 17.
21 C. H. CHERVENKA AND P. E. WILCOX, *J. Biol. Chem.*, 222 (1956) 621.
22 W. A. KLEE AND F. M. RICHARDS, *J. Biol. Chem.*, 229 (1957) 489.
23 S. LEWIN, *J. Theoret. Biol.*, 23 (1969) 279.